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(54) Title: DETECTION OF HUMAN PAPILLOMAVIRUS BY THE POLYMERASE CHAIN REACTION

(57) Abstract

The presence of human papillomavirus (HPV) in a sample can be detected and the HPV typed by a method that involves the amplification of HPV DNA sequences by the polymerase chain reaction (PCR). The primers used in the method are consensus primers that can be used to amplify a particular region of the genome of any HPV. The presence of HPV in a sample is indicated by the formation of amplified DNA. The HPV is typed by the use of type-specific DNA probes specific for the amplified region of DNA .

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Detection of Human Papillomavirus by the Polymerase Chain Reaction

The present invention provides medical research and diagnostic methods for detecting and typing HPV. The method utilizes PCR, a DNA amplification technique widely used in the fields of molecular biology and genetic engineering. The method can also be used to generate information concerning previously unknown strains of HPV and consequently has applications in the field of virology.

Papillomaviruses have been linked to widespread, serious human diseases, especially carcinomas of the genital and oral mucosa. And although genital HPV infection is associated with cancer primarily in women, recent evidence suggests that HPV may play a role in the development of prostate cancer in men. Broker et al., 1986, Cancer Cells 4:17-36, review the molecular, cellular, and clinical aspects of the papillomaviruses and the relationship of HPVs to cancer. HPV types 6, 11, 16, 18, and 33 are known genital HPV types in the human population, and Broker et al., 1986, Cancer Cells 4:589-594, disclose that HPV types 6, 11, 16, 18, and 33 share significant homology at the DNA level, particularly at the L1 open reading frame.

Identification and typing of HPV is quite important, because different types of HPV pose different risks to the affected individuals. For instance, HPV16 and HPV18 have been more consistently identified in higher grades of cervical dysplasia and carcinoma than other HPV types. Webb et al., December 1987, J. Inf. Disease 156(6):912-919, report a method for detecting HPV DNA types that utilizes a reverse-blotting procedure. The procedure involved forming a membrane to which genomic DNA from four different HPV types was bound and then hybridizing labelled DNA from a biological sample to the DNA bound to the membrane. Caussey et al., February 1988, J. Clin. Microbiol. 26(2):236-243 describe similar HPV detection methods.

Shibata et al., January 1988, J. Exp. Med. 167:225-230, disclose the use of PCR to amplify and detect the presence of HPV16 and HPV18 DNA. U.S. Patent Nos. 4,683,195 and 4,683,202 disclose PCR and the use of PCR to detect the presence or absence of nucleic acid sequence in a sample. European Patent Publication Nos. 229,701 and 269,445 disclose the use of PCR to amplify and detect DNA sequences associated with a wide variety of viruses, including the AIDS virus, HTLV I, and HTLV II.

Maitland et al., May 1988, Seventh International Papillomavirus Workshop, Abstract, p. 5, report the use of PCR to detect HPV16 in oral and cervical biopsies. In addition, Campione-Piccardo et al., May 1988, Seventh International Papillomavirus Workshop, Abstract, p. 19, report the use of a mixture of primers for the specific

amplification by PCR of HPV sequences in types 1a, 5, 6a, 6b, 8, 11, 16, 18, and 33. A number of other researchers disclosed the use of PCR to amplify and detect HPV sequences at the Seventh International Papillomavirus Workshop.

Despite the use of PCR to amplify and detect HPV sequences, there still remains a need for a simple and rapid method for both detecting and typing HPV in a biological sample. The present invention provides a method that meets that need.

The present invention provides a method for detecting and typing HPV in a sample. The method comprises amplifying a sequence of HPV DNA present in the sample, determining if amplification has occurred, and then hybridizing an HPV type-specific probe to the amplified DNA. The invention also provides novel primers and probes for use in the method.

The present invention provides a method for detecting HPV in a sample, and typing the HPV if present, the method comprising:

(a) treating the sample with consensus HPV primers, an agent for polymerization, and deoxynucleoside 5'-triphosphates under conditions such that an extension product of a consensus primer can be synthesized, wherein said consensus primers are a mixture of oligonucleotides that comprises at least a pair of primers sufficiently complementary to separate single strands of HPV DNA to hybridize thereto so that the extension product synthesized from one member of said pair, when separated from its complementary strand, can serve as a template for synthesis of the extension product of the other member of said pair;

(b) separating the primer extension products, if present, from the templates on which the extension products were synthesized to form single-stranded molecules;

(c) treating the single-stranded molecules generated in step (b), if any, with the consensus primers of step (a) under conditions such that a primer extension product is synthesized using each of the single-stranded molecules produced in step (b) as a template;

(d) repeating steps (b) and (c) at least once;

(e) determining if amplification has occurred; and if amplification has occurred,

(f) hybridizing an HPV type-specific DNA probe to said amplified DNA; and

(g) determining if hybridization has occurred.

The first step in the method of the invention requires the use of consensus primer pairs that will amplify a discrete region of DNA from HPV DNA present in a sample. These consensus primer pairs are oligonucleotides, and the consensus primers are mixtures of primer pairs. The mixtures used in the method assure that, regardless of the type of HPV present in a sample, HPV DNA corresponding to the region between the "consensus" sequences will be amplified. The PCR products generated

from the consensus primers, if HPV is present in the sample, are then analyzed by hybridization with type-specific probes to determine the HPV types present.

Amplification of DNA by the polymerase chain reaction (PCR) is disclosed in U.S. Patent Nos. 4,683,202 and 4,683,195 and in the related applications noted and incorporated by reference in the Cross-Reference, above. PCR amplification of DNA involves repeated cycles of heat-denaturing the DNA, annealing two oligonucleotide primers to sequences that flank the DNA segment to be amplified, and extending the annealed primers with DNA polymerase. The primers hybridize to opposite strands of the target sequence and are oriented so DNA synthesis by the polymerase proceeds across the region between the primers, effectively doubling the amount of that DNA segment. Moreover, because the extension products are also complementary to and capable of binding primers, each successive cycle essentially doubles the amount of DNA synthesized in the previous cycle. This results in the exponential accumulation of the specific target fragment, at a rate of approximately 2n per cycle, where n is the number of cycles.

The choice of primers for use in PCR determines the specificity of the amplification reaction. In the amplification steps of the method of the present invention, "consensus" primers are used that will amplify genital HPV sequences present in a sample, regardless of type. The consensus primers of the invention can include degenerate primers, mixtures of the oligonucleotides synthesized so that any one of several nucleotides can be incorporated into a primer at a selected position during synthesis. The consensus primers are sufficiently complementary to all types of HPVs to amplify a DNA sequence of any HPV present in the sample. The consensus primers are also designed to amplify a region of DNA that contains sequences that are specific to each major viral type, so the amplified DNA can therefore be used to type the HPV present in the sample.

The invention, although applicable to any HPV, is exemplified below with reference to genital HPV strains. Furthermore, the primers and probes of the invention can be targeted to areas of the HPV genome other than those described below, provided that the particular area targeted can be amplified using consensus primers and the amplified DNA can be typed using type-specific probes.

The first step of the method of the present invention involves the amplification of an HPV sequence, if that sequence is present in a sample, by PCR using consensus primers. Illustrative consensus primers of the invention are referred to by the region of the HPV genome the primers are used to amplify. The HPV genome is circular. The genome of genital HPVs is oriented as follows: E6, E7, E1, E2, E4, E5a, E5b, L2, L1, and URR. "E" and "L" designations indicate open reading frames, but many of the

open reading frames overlap. For instance, E4 is totally contained within the E2 open reading frame. URR is the transcriptional regulatory region. Primers can be used to amplify a sequence that spans one or more regions of the HPV genome.

For instance, the L1/E6 consensus primer combinations of the invention are designed to amplify a sequence of DNA from any genital HPV. The amplified sequence extends from L1 across the URR and into E6 and thus contains portions of the L1 and E6 regions with the URR region sandwiched in between the L1 and E6 regions. Thus, the consensus primer pairs consist of a first primer specific for a sequence in the L1 region and a second primer specific for a sequence in the E6 region. As shown in Table 1, below, the first L1-specific primer can be either FS10, FS17, or MY01, while the second, E6-specific primer is at least a 1:1 mixture of JS15 and JS16, although the mixture can also contain more JS15 than JS16. Table 1 also depicts the sequence each primer and the corresponding sequence (and nucleotide position of that sequence) as it occurs in the genomes of several well-known genital HPVs (Types 6, 11, 16, 18, and 33). A dash in a sequence indicates that the genomic sequence is identical to the primer sequence. Nucleotides are abbreviated as follows:

Symbol	Meaning	Origin
G	G	Guanine
A	A	Adenine
T	T	Thymine
C	C	Cytosine
R	G or A	puRine
Y	T or C	pYrimidine
M	A or C	aMino
K	G or T	Keto
S	G or C	Strong interaction (3 H bonds)
W	A or T	Weak interaction (2 H bonds)
H	A or C or T	not-G, H follows G in the alphabet
B	G or T or C	not-A, B follows A
V	G or C or A	not-T (not-U), V follows U
D	G or A or T	not-C, D follows C
N	G or A or T or C	aNy

Table 1

L1/E6 Consensus Primers and Amplification Products

L1 Consensus Positive Strand Primers

FS10	25mer	5' C T G T G G T A G A T A C C A C A C G C A G T A C
HPV06	6770	- -
HPV11	6756	- -
HPV16	6633	- - - - T - - T - - - - - T - - - - - - - - - - -
HPV18	6587	- - - - - - - - - - - - - - - - T - - - - - - - -
HPV33	6587	- - - - - - - - - - - - - - - - T - - - - - - - -
FS17	20mer	5' G A T C A G T T C C Y Y T K G G A C G
MY01	20mer	5' G A T C A G T W T C C Y Y T K G G A C G
HPV06	7151	- - - - - - - - A - - - T T - G - - - - -
HPV11	7136	- - - - - - - - T - - - C C - T - - - - -
HPV16	7015	- - - - - - - - T - - - T T - A - - - - -
HPV18	6993	- - - - - - A - A - - - C C - T - - - - -
HPV33	6968	- - - - - - - - T - - - T T - G - - - - -

URR/E6 Consensus Negative Strand Primer

JS15	18mer	5' C C G T T T T C G G T T S A A C C G
HPV06	60	- - - - - - - - - - - - - - G - - - - -
HPV11	60	- - - - - - - - - - - - - - G - - - - -
HPV16	60	- - - G - - - - - - - - - - C - - - - -
HPV33	64	- - - - - - - - - - - - - - G - - - - -
JS16	19mer	5' C C G T T T T C G G T C C C G A C C G
HPV18	68	- - - - - - - - - - - - - - - - - - - -

Predicted Sizes Of PCR Products

+ PRIMER	FS10	FS17 or MYO1
- PRIMER	JS15 and JS16	JS15 and JS16
HPV06	1192 bp	822 bp
HPV11	1235 bp	856 bp
HPV16	1387 bp	958 bp
HPV18	1367 bp	932 bp
HPV33	1434 bp	1005 bp

As shown in Table 1, FS10 is a 25-mer that has 3 mismatches with HPV16 and 1 mismatch with HPV18 and HPV33. FS17 is a degenerate primer with 1 or 2 mismatches to different HPVs. MY01 is similar to FS17, contains 1 more degenerate base to decrease mismatches and to potentially cover a wider range of HPVs. JS15 is a

degenerate 18-mer for the negative strand in E6 of HPVs 6, 11, 16, and 33, whereas JS16 is a 19-mer serving the same function for HPV18.

Once a sample has been treated with the L1/E6 primers shown above under conditions suitable for PCR, the method of the invention requires the determination of whether amplification has occurred. If amplification has occurred with the L1/E6 primers, HPV sequences are present in the sample. The use of an internal amplification control to assure the competency of a sample for PCR is within the scope of the invention and reduces the likelihood of false negative results. There are a variety of ways to determine whether amplification has occurred. A portion of the reaction mixture can be subjected to gel electrophoresis and the resulting gel stained with ethidium bromide and exposed to ultraviolet light to observe whether a product of the expected size is present. Labeled primers or deoxyribonucleotide 5'-triphosphates can be added to the PCR reaction mixture, and incorporation of the label into the amplified DNA is measured to determine if amplification occurred. Another method for determining if amplification has occurred is to test a portion of PCR reaction mixture for ability to hybridize to a labeled oligonucleotide probe designed to hybridize to only the amplified DNA. The probe must be a consensus probe so that amplified DNA from any HPV can be detected. For instance, amplification of HPV DNA using the L1/E6 consensus primers FS10, JS15, and JS16 can be detected using the L1/E6 consensus primer FS17 or MY01. Alternatively, the determination of amplification and identification of HPV type can be carried out in one step by testing a portion of the PCR reaction mixture for its ability to hybridize to one or more type-specific probes.

An important aspect of the present invention relates to the novel probes provided for use in the present methods. Whether these probes are consensus probes for determining if amplification has occurred or whether these probes are type-specific probes, the probes can be used in a variety of different hybridization formats. Although solution hybridization of a nucleic acid probe to a complementary target sequence is clearly within the scope of the present invention, commercialization of the invention will likely result in the use of immobilized probes and thus a quasi "solid-phase" hybridization. In this format, the probe is covalently attached to a solid support and then the target sequences are then hybridized with the probe. In this method, the probes are attached to a solid support by virtue of long stretches of T residues; these T residues are added to the probe during synthesis of the probe on an automated synthesizer after the hybridizing sequence is synthesized. A variety of dyes and chromogens and corresponding labels are available for nucleic acid detection systems.

The present invention has led and will continue to lead to the discovery of so many previously unknown (or at least uncharacterized) HPV types, however, that the

embodiment of the invention in which consensus probes are used to determine if amplification has occurred will continue to decline in importance. This is because that with each new type of HPV discovered there comes a corresponding need to make the consensus probe more generic to ensure that the new type will be detected. To overcome this problem, the present invention provides a new type of consensus probe. This new type of probe essentially consists of a sequence of DNA from one or more HPV viruses that comprises all or most of the DNA sequence that lies between the two sequences on the HPV genome corresponding to the primers used in the amplification. The consensus probe should not, however, comprise a sequence complementary to either primer used to amplify the HPV DNA in the sample. This new type of consensus probe has greater versatility in that it has more sequence available for hybridization than the other consensus probes of the invention. In addition, the consensus probe can be generated with PCR with primers that hybridize to sequences that lie inside the region of DNA defined by the primers used to amplify the HPV DNA sequences present in a sample.

The present invention provides a number of type-specific probes for use with the L1/E6 consensus primers of the invention. These probes are set forth in Table 2, below. Those skilled in the art will recognize that although the specific primers and probes of the invention exemplified herein have a defined number of nucleotide residues, one or more nucleotide residues may be added or deleted from a given primer or probe typically without great impact on the suitability of that primer or probe in the present methods.

Table 2

HPV Typing Probes For Use with L1/E6 Consensus Primers

Specificity	Sequence	Size	Designation
HPV6	5'CCAAACAGTAAGAGC	(15-mer)	FS18
HPV11	5'GGCTGTAGAGGGCTTAGAC	(19-mer)	FS19
HPV16	5'GGTTGAAGCTACAAAATGGGCC	(22-mer)	JS17
HPV18	5'GTAGCGCACCTGGACAGG	(18-mer)	FS21
HPV33	5'CAGGTAGTGACTCAC	(15-mer)	FS22

FS19 and JS17 can specifically detect HPV11 and HPV16, respectively. FS18 shows some hybridization with the HPV11 PCR product. UWGCG GAP program analysis comparing FS18 sequence and HPV11 sequence indicates a 73% homology of FS18 to HPV11 in the amplified region. The cross-hybridization could be minimized by increasing the stringency of washing. FS21 was specific for HPV18.

The L1/E6 primers disclosed above provide for the amplification of relatively large segments of HPV DNA. However, use of primers that result in shorter PCR products can have several advantages, including reduced extension and denaturation time and decreased denaturation temperature. The L1 consensus primers of the invention are illustrative of primers of the invention designed to amplify relatively small segments of the HPV genome to achieve such advantages. The L1 consensus primers produce a PCR product corresponding to sequences in the L1 open reading frame and are depicted in Table 3, below.

Table 3

L1 Consensus Primers and Amplification Products

L1 Consensus Positive Strand Primer

MY11	20mer	5'	G C M C A G G G W C A T A A Y A A T G G
HPV06	6722		- - C - - - - - A - - - - - C - - - - -
HPV11	6707		- - T - - - - - A - - - - - C - - - - -
HPV16	6582		- - A - - - - - C - - C - - T - - - - -
HPV18	6558		- - A - - - - - T - - - - - C - - - - -
HPV33	6539		- - A - - A - - T - - - - - T - - - - -

L1 Consensus Negative Strand Primer

MY09	20mer	5'	C G T C C M A R R G G A W A C T G A T C
HPV06	7170		- - - - - C - A A - - - T - - - - - - -
HPV11	7155		- - - - - A - G G - - - A - - - - - - -
HPV16	7033		- - - - - T - A A - - - A - - - - - - -
HPV18	6712		- - - - - A - G G - - - T - T - - - - -
HPV33	6987		- - - - - C - A A - - - A - - - - - - -

Predicted sizes of PCR products from the MY11 and MY09 L1 Consensus Primer Pair

HPV06	448 bp
HPV11	448 bp
HPV16	451 bp
HPV18	454 bp
HPV33	448 bp

A preferred embodiment of the method of the present invention for genital HPVs comprises amplification of HPV sequences, if present in the sample, with the L1 consensus primers MY09 and MY11; determination of amplification by hybridization of a portion of the PCR reaction mixture with a generic genital HPV probe; and finally,

type determination with type-specific probes. To determine if amplification of HPV DNA sequences has occurred in a sample that has been treated with the L1 consensus primers of the invention, a portion of the PCR reaction mixture can be hybridized with L1 consensus probes, depicted in Table 4.

Table 4

L1 Consensus Probes

FS10	5' C T G T G G T A G A T A C C A C A C G C A G T A C
MY18	5' - - - - T - - T - - - - - T - - - - - - - - - - -
MY19	5' - - - - - - - - - - - - - - - - - T - - - - - - -

Sequence of HPV types in Region of Consensus Probe

HPV6	6771	5' C T G T G G T A G A T A C C A C A C G C A G T A C
HPV11	6756	5' -
HPV16	6631	5' - - - - T - - T - - - - - T - - - - - - - - - - -
HPV18	6607	5' - - - - - - - - - - - - - - - - - T - - - - - - -
HPV33	6588	5' - - - - - - - - - - - - - - - - - T - - - - - - -
Isolate	36	5' - - - - T - - G - - - - - T - - C A - A - - C - -
Isolate	88	5' - A - - - - - - - - - - - T - - T - - - - - C - -

When a portion of the PCR reaction mixture contains DNA that hybridizes to a probe contained in the L1 consensus probe mixture, the sample contains HPV DNA. There are a number of ways to determine whether a probe has hybridized to a DNA sequence contained in a sample. Typically, the probe is labeled in a detectable manner, the target DNA (i.e., the amplified DNA in the PCR reaction buffer) is bound to a solid support, and determination of whether hybridization has occurred simply involves determining whether the label is present on the solid support. This procedure can be varied, however, and works just as well when the target is labeled and the probe is bound to the solid support. Many methods for labelling nucleic acids, whether probe or target, are known in the art and are suitable for purposes of the present invention. In the laboratory work involved in reducing the present invention to practice, probes were typically labeled with radioactive phosphorous ^{32}P, by treating the probes with polynucleotide kinase in the presence of radiolabelled ATP. For commercial purposes, however, other non-radioactive labeling systems may be preferred, i.e., horseradish peroxidase-avidin-biotin systems.

Whatever the labeling system used, once a determination has been made that the L1 consensus probe has hybridized to amplified DNA present in the sample, the amplified DNA is typed to determine the HPV types present in the sample. Practice of the present invention has led to the discovery of many previously uncharacterized HPV types. For example, three clinical samples examined by the present method contained

five different HPV types with sequences markedly different than the published sequences for HPVs. These new sequences are an important aspect of the present invention, as are the probes that will hybridize to these sequences in a type-specific fashion. These new sequences are depicted below. Degenerate nucleotides are as defined above and correspond to the degenerate nucleotides in the primers used to amplify the region or to variation within the type.

The DNA Sequence of the L1 Amplified Regions of HPV Isolates 36A, 36B, 88, 238A, and 238B

Isolate 36A

```
  1  GCMCAGGGWC ATAAYAATGG TATATGTTGG CACAATCAAT TGTTTTTAAC
 51  AGTTGTAGAT ACTACTCGCA GCACCAATCT YTCTGTGTGT GCTTCTACTA
101  CTTCTCCTAT TCCTAATGAA TACACACCTA CCAGTTTTAA AGAATATGCC
151  AGACATGTGG RGGAATTTGA TTTGCAGTTT ATAYTTCAAC TGTGTAAAAT
201  AACWTTAACT ACAGAGGTAA TGTCATACAT TCATAATATG AATACCACTA
251  TTTTGGAGGA TTGGAATTTT GGTRTTACAC CACCTCCTAC TGCTARTTTA
301  GTTGACACAT ACCGTTTTGT TCAATCTGCT GCTGTAACTT GTCAAAAGGA
351  CACCGCACCG CCAGTTAAAC AGGACCCTTA TGACAAACTA AAGTTTTGGA
401  CTGTAAATCT TAAGGAAAGG TTTTCTGCAG ATCTTGATCA GTWTCCYYTK
451  GGACG
```

Isolate 36B

```
  1  GCMCAGGGWC ATAAYAATGG TATATGTTGG GGAAATCAGC TATTTTTAAC
 51  TGTGGTTGAT ACTACCCGTA GTACTAACAT GACTTTGTGY GCCACTGCAA
101  CATCTGGTGA TACATATACA GCTGCTAATT TTAAGGAATA TTTAAGACAT
151  GCTGAAGAAT ATGATGTGCA ATTTATATTT CAATTGTGTR AAATAACATT
201  AACTGTTGAA GTTATGTCAT ATATACACAA TATGAATCCT AACATATTAG
251  AGGAGTGGAA TGTTGGTGTT GCACCACCAC CTTCAGGAAC TTTAGAAGAT
301  AGTTATAGGT ATGTACAATC AGAAGCTATT CGCTGTCAGG CTAAGGTAAC
351  AACGCCAGAA AAAAAGGATC CTTATTCAGA CTTTTCCTTT TGGGAGGTAA
401  ATTTATCTGA AAAGTTTTCT ACTGATTTAG GATCAGTWTC CYYTKGGACG
```

Isolate 88

```
   1  GCMCAGGGWC ATAAYAATGG CATATGCTGG GGTAATCAGG TATTTGTTAC
  51  TGTTGTGGAT ACTACCAGAA GCACCAACAT GACTATTAAT GCAGCTAAAA
 101  GCACATTARC TAAATATGAT GCCCGTGAAA TCAATCAATA CCTTCGCCAT
 151  GTGGAGGAAT ATGAACTACA GTTTGTGTTT CAACTTTGTA AAATAACCTT
 201  AACTGCAGAR GTTATGGCAT ATTTGCATAA TATGAATAAT ACTTTATTRG
 251  ACGATTGGAA TATTGGCTTA TCCCCACCAG TTGCAACTAG CTTAGAGGAT
 301  AAATATAGGT ATATTAAAAG CACAGCTRTT ACAYGTCAGA GGGAACAGCC
 351  CCCTGCAGAA AAGCAGGATC CCCTGGCTAA ATATAAGTTT TGGGAAGTTA
 401  ATTTACAGGA CAGCTTTTCT GCAGACCTGG GATCAGTWTC CYYTKGGACG
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Isolate 238A

```
   1  GCMCAGGGWC ATAAYAATGG TATTTGTTGG CATAATCART TATTTTTAAC
  51  TGTTGTAGAT ACTACTAGAA GCACTAATTT TTCTGTATGT GTAGGTACAC
 101  AGGCTAGTAG CTCTACTACA ACGTATGCCA ACTCTAATTT TAAGGAATAT
 151  TTAAGACATG CAGAAGAGTT TGATTTACAG TTTGTTYTTC AGTTATGTAA
 201  AATTAGTTTA ACTACTGAGG TAATGACATA TATACATTCT ATGAATTCTA
 251  CTATATTGGA AGAGTGGAAT TTTGGTCTTA CCCCACCACC GTCAGGTACT
 301  TTAGAGGAAA CATATAGATA TGTAACATCA CAKGCTATTA GTTGCCAACG
 351  TCCTCAACCT CCTAAAGAAA CAGAGGACCC ATATGCCAAG CTATCCTTTT
 401  GGGATGTAGA TCTTAAGGAA AAGTTTTCTG CAGAATTAGA TCAGTWTCCY
 451  YTKGGACG
```

Isolate 238B

```
   1  GCMCAGGGWC ATAAYAATGG TATTTGTTGG GGCAATCAGT TATTTGTTAC
  51  TGTGGTAGAT ACCACACGTA GTACCAATAT GTCTGTGTGT GCTGCAATTG
 101  CAAACAGTGA TACTACATTT AAAAGTAGTA ATTTTAAAGA GTATTTAAGA
 151  CATGGTGAGG AATTTGATTT ACRATTTATA TTTCAGTTAT GCAAAATAAC
 201  ATTATCTGCA GACATAATGA CATATATTCA CAGTATGAAT CCTGCTATTT
 251  TGGAAGATTG GAATTTTGGA TTGACCACAC CTCCCTCAGG TTCTTTAGAG
 301  GATACCTATA GGTTTGTAAC CTCACAGGCC ATTACATGTC AAAAARCTGC
 351  CCCCCAAAAG CCCAAGGAAG ATCCATTTAA AGATTATGTA TTTTGGGAGG
 401  TTAATTTAAA AGAAAAGTTT TCTGCAGATT TAGATCAGTW TCCYYTKGGA
 451  CG
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Isolate 155A and 155B

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   1  TATATGCTGG TTTAATCAAT TGTTTGTCAC GGTGGTGGAT ACCACCCGCA
  51  GCACCAATTT TACTATTAGT GCTGCTACCA ACACCGAATC AGAATATAAA
 101  CCTACCAATT TTAAGGAATA CCTAAGACAT GTGGAGGAAT ATGATTTGCA
 151  GTTTATATTC CAGTTGTGTA AGGTCCGTCT GACTCCAGAG GTCATGTCCT
 201  ATTTACATAC TATGAATGAC TCCTTATTAG ATGAGTGGAA TTTTGGTGTT
 251  GTGCCCCCTC CCTCCACAAG TTTAGATGAT ACCTATAGGT ACTTGCAGTC
 301  TCGCGCCATT ACTTGCCAAA AGGGGGCCGC CGCCGCCAAG CCTAAGGAAG
 351  ATCCTTATGC TGGCATGTCC TTTTGGGATG TAGATTTAAA GGACAAGTTT
 401  TCTACTGATT TG
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Isolate C14

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   1  TATTTGTTGG CATAATCAGT TGTTTGTTAC TGTAGTGGAC ACTACCCGCA
  51  GTACTAATTT AACATTATGT GCCTCTACAC AAAATCCTGT GCCAAATACA
 101  TATGATCCTA CTAAGTTTAA GCACTATAGT AGACATGTGG AGGAATATGA
 151  TTTACAGTTT ATTTTTCAGT TGTGCACTAT TACTTTAACT GCAGAGGTTA
 201  TGTCATATAT CCATAGTATG AATAGTAGTA TATTGGAAAA TTGGAATTTT
 251  GGTGTACCTC CACCACCTAC TACAAGTTTA GTGGATACAT ATCGTTTTGT
 301  GCAATCCGTT GCTGTTACCT GTCAAAAGGA TACTACACCT CCAGAAAAGC
 351  AGGATCCATA TGATAAATTA AAGTTTTGGA CTGTTGACCT AAAGGAAAAA
 401  TTTTCCTCCG ATTTG
```

Those skilled in the art will recognize that with the above sequence information, primers and probes for amplifying and detecting these new HPV isolates can be readily obtained. In addition, these sequences enable one to isolate the entire virus from

samples containing the virus. Isolate 238B corresponds to the HPV31 type described in the literature. A cervical carcinoma isolate, C14, is a variant of HPV45. The discovery of these new HPV isolates led to the creation of additional L1 consensus probes for use in conjunction with FS10, MY18, and MY19. These L1 consensus probes are depicted below under the FS10 probe sequence to demonstrate the similarity to the FS10 sequence. L1 consensus probe WD147 will hybridize to HPV45 DNA.

FS10	5' C T G T G G T A G A T A C C A C A C G C A G T A C
MY66	- A - - T - - - - - - - - T - - T - - - - - C - -
MY55	- - - - - - - T - - - - - - T - - C - - T - - - - -
MY39	- - - - T - - G - - - - - - T - - C A - A - - C - -
MY56	- - - - T - - - - - - - - - T - - T A - A - - C - -
MY57	- - - - - - - - - - - - - - - - - - - T - - - - -
WD147	- - - - A - - G - - C - - T - - C - - - - - - - -

As noted above, the diversity of HPV types may mandate the use of longer consensus probes that contain almost all of the amplified sequence except that portion corresponding to the primers used in the amplification step of the present method. This diversity in HPV types also demonstrates the need for the type-specific probes provided by the present invention.

The present invention provides a number of probes for typing amplified DNA produced from L1 consensus primers. These probes are depicted below in Table 5.

Table 5

HPV Typing Probes For Use with L1 Consensus Primers

Probe	Specificity	Sequence	Genome Position
MY12	HPV6	5'CATCCGTAACTACATCTTCCA	6813-6833
MY13	HPV11	5'TCTGTGTCTAAATCTGCTACA	6800-6820
MY14	HPV16	5'CATACACCTCCAGCACCTAA	6926-6945
WD74	HPV18	5'GGATGCTGCACCGGCTGA	6905-6922
MY16	HPV33	5'CACACAAGTAACTAGTGACAG	6628-6648
MY58	HPV16	5'TTGTAACCCAGGCAATTGCT	6897
MY59	HPV33	5'AAAAACAGTACCTCCAAAGGA	6877
MY60	HPV18	5'CAGTCTCCTGTACCTGGG	6657
MY61	HPV11	5'CACACCTGAAAAAGAAAAACAG	7051
MY62	HPV6	5'CTCCTGAAAAGGAAAAGCCA	7068
MY63	HPV6	5'TGGCTTTTCCTTTTCAGGAG	7068
MY64	HPV33	5'TCCTTTGGAGGTACTGTTTTT	6877
MY65	HPV11	5'CTGTTTTTCTTTTTCAGGTGTG	7051
WD126	HPV31	5'CCAAAAGCCCAAGGAAGATC	N.D.
WD127	HPV31	5'CAAAAGCCCAAGGAAGATC	N.D.
WD128	HPV31	5'TTGCAAACAGTGATACTACATT	N.D.
MY69	HPV45	5'ATACTACACCTCCAGAAAAGC	N.A.
MY70	HPV45	5'TAGTGGACACTACCCGCAG	N.A.
WD150	HPV 11	5'CAGAAACCCACACCTGAAAAAGA	7059
WD151	HPV11	5'AGAAACCCACACCTGAAAAAGAA	7058
WD152	HPV16	5'TTTGTAACCCAGGCAATTGCT	6898
WD153	HPV11	5'GTTTGTAACCCAGGCAATTGCT	7053

N.D.: Not determined
N.A.: Not available

The present invention also provides consensus primers and HPV typing probes for detection of DNA sequences specific for the E6 region of genital HPVs. These probes are particularly preferred, because in some HPV-infected individuals, the HPV genome is partially deleted or rearranged such that only E6- and E7-related sequences are present. The E6 consensus primer pairs of the invention comprise primer pairs in which one primer is complementary to sequences near the border of the URR and E6 regions and the other primer is complementary to sequences in either the E7 region near the E6-E7 border (the E6 and E7 open reading frames overlap) or in the E6 region. These E6 consensus primers are depicted below in Table 6.

Table 6

E6 Consensus Primers

URR/E6 Consensus Positive Strand Primers

	WD73		5' C G G T T S A A C C G A A A A C G G
	WD72		5' C G G T C G G G A C C G A A A A C G G
	WD76		5' C G G T T S A A C C G A A A M C G G
	WD77		5' C G G T T C A A C C G A A A M C G G
	HPV06	43	5' C G G T T C A A C C G A A A A C G G
	HPV11	43	- - - - - - - - - - - - - - - - - - -
	HPV16	43	- - - - - G - - - - - - - - C - - -
	HPV18	43	- - - - C G G G - - - - - - - - - - -
	HPV33	65	- - - - - - - - - - - - - - - - - - -

The URR/E6 positive strand primers are used as mixtures: WD72 and WD73; WD72 and WD76; and WD72 and WD77.

E7 Consensus Negative Strand Primer

	WD70		5' G C R C A G A T G G G R C A C A C
	WD71		5' G C A C A C C A C G G A C A C A C
	HPV06	813	5' G C G C A G A T G G G A C A C A C
	HPV11	813	- - - - - - - - - - - - - - - - - - -
	HPV16	845	- A A - - - - - - - - - G - - - - -
	HPV18	894	- - A - - C C A C - - - - - - - - -
	HPV33	856	- - A - - - G - A - - G - - - - - -
	WD68		5' C A C A C A A T D Y Y Y A G T G T G C C C
	WD69		5' C A C A C A A A G G A C A G G G T G T T C
	HPV06	801	5' C A C A C T A T G T T T A G T G T T C C C
	HPV11	801	- - - - - - A - - A - - - - - - - - G - - -
	HPV16	833	- - - - - - A - - T C C - - - - - - - G - - -
	HPV18	882	- - - - - - A - A - G A C - - G - - G T T -
	HPV33	844	- - - - - - A - - A - - C - C - - - G - - -

The E7 negative strand primers are used as mixtures: WD70 and WD71; and WD68 and WD69.

Table 6 (continued)

E6 Consensus Negative Strand Primer

	WD67		W G C A W A T G G A W W G C Y G T C T C
	WD66		A G C A T G C G G T A T A C T G T C T C
5	WD154		T C C G T G T G G T G T G T C G T C C C
	WD155		W S C A W A T G G W W W G Y C G T C Y C
	WD163		W G C A W A T G G A W W G Y Y G T C Y C
	WD164		W S C A W A T G G W D W G Y Y G T C Y C
10	HPV6	286	T G C A T A T G G A T A G C C G C C T C
	HPV11	286	- - - - A - G - - - A - - T T - T - - -
	HPV16	286	A - - - - - - - - - T C - - - A T - - -
	HPV18	292	A - - - - G C - - T A T A - T - T - - -
	HPV31		- C - G - G - - - T G T - T - - T - C -
15	HPV33	390	- C - - A - - - - - - T T - - C T - - -

Additional E6 consensus negative strand primers located in a different region of the gene, are used in pairs as shown below. The three primer sets shown, WD157 and WD160; WD158 and WD161; and WD159 and WD162, each correspond to the same genomic HPV region, but they differ in length. Primers WD160, WD161, WD162 are
20 HPV18 specific.

WD157	TTCTAMTGTWGTTSCATAYACASHATA
WD160	CCAATGTGTGTCTCCATACACAGAGTC
WD158	CTAMTGTWGTTSCATAYACASHATA
WD161	AATGTGTGTCTCCATACACAGAGTC
25 WD159	TAMTGTWGTTSCATAYACASHATA
WD162	ATGTGTGTCTCCATACACAGAGTC

Predicted Sizes of Products from E6 Consensus Primers

URR/E6 Primer	E7 Primer	Product Size for HPV Type				
		HPV6	HPV11	HPV16	HPV18	HPV33
WD72 and WD73	WD70 and WD71	770	770	802	844	791
30 WD72 and WD76	WD70 and WD71	770	770	802	844	791
WD72 and WD77	WD70 and WD71	770	770	802	844	791
WD72 and WD73	WD68 and WD69	758	758	790	832	779
WD72 and WD76	WD68 and WD69	758	758	790	832	779
WD72 and WD77	WD68 and WD69	758	758	790	832	779
35 WD72 and WD73	WD66 and WD67	243	243	242	242	225
WD72 and WD76	WD66 and WD67	243	243	242	242	225
WD72 and WD77	WD66 and WD67	243	243	242	242	225

Those skilled in the art will recognize that the E6 consensus primers of the invention amplify a sequence that comprises a portion of E7 DNA. To determine if

amplification has occurred when the E6 consensus primers are used in the method of the invention, E6 consensus probes are provided. E6 consensus probes WD134 and WD135 are directed to the small E6 amplification product and are used together as a mixture. The E6 consensus probes can also be used as E6 consensus positive strand primers. When used as E6 consensus primers, the E6 consensus probes are used in the following combinations: WD65 and WD64; WD83 and WD64; and WD84 and WD64. The E6 consensus probes are depicted in Table 7.

Table 7

E6 Consensus Probes

WD65		M G A G A C R G C W W T C C A T W T G
WD83		M G A G A C R G S W W T C C A T W T G
WD84		M G A G A C R G V W W T C C A T W T G
WD64		A G A G A C A G T A T A C C G C A T G
HPV6	267	C G A G G C G G C T A T C C A T A T G
HPV11	267	- - - - A - A A - - T - - - C - T - -
HPV16	266	A - - - A T - - G A - - - - - - - - -
HPV18	273	A - - - A - A - T A T A - - G C - -
HPV33	271	A - - - A G - - A A - - - - - - T - -
WD134		G A G G T A T W T G A H T T T G C
WD135		G A G A T W T A T K C A T A T G C

To determine the type of the HPV present in a sample when the E6 consensus primers are used in the method of the invention, E6 type-specific probes are provided. These probes are depicted in Table 8. Using URR/E6 consensus primers comprising positive strand primers of Table 6 with WD70 and WD71 or WD68 and WD69 any of the HPV typing probes of Table 8 or Table 8A will be effective. These typing probes are also useful when E1 negative strand primers are used for amplification with the URR/E6 positive strand consensus primers.

Table 8

HPV Typing Probes for Use with E6 Amplified Sequences

Probe	Specificity	Sequence	Genome Position
WD78	HPV6	5' CGAAGTGGACGGACAAGAT	643
WD79	HPV11	5' CAAGGTGGACAAACAAGACG	643
WD80	HPV16	5' GAACACGTAGAGAAACCCAG	534
WD81	HPV18	5' CAACCGAGCACGACAGGA	530
WD82	HPV33	5' GAGGTCCCGACGTAGAGAA	534

Table 8A

HPV Typing Probes for Use with Small E6 Amplified Sequences

Probe	Specificity	Sequence	Genome Position
WD165	HPV31	5' AAATCCTGCAGAAAGACCTC	
WD166	HPV31	5' CCTACAGACGCCATGTTCA	
WD167	HPV39	5' CCTTGCAGGACATTACAATAG	
WD168	HPV39	5' CAGACGACCACTACAGCAA	
WD169	HPV42	5' GGTGCAAAAAGCATTAACAG	
WD102	HPV18	5' ACAGTATTGGAACTTACAG	213
WD103	HPV16	5' CAACAGTTACTGCGACG	206
WD104	HPV33	5' GCAGTAAGGTACTGCAC	88
WD132	HPV18	5' GACAGTATTGGAACTTACAG	213
WD133	HPV6	5' ACACCTAAAGGTCCTGTTTC	248
WD134	HPV11	5' ACACTCTGCAAATTCAGTGC	175

The typing probes of Table 8A are useful with the positive strand URR/E6 consensus primers selected from WD76, WD66 and WD154; WD157 and WD160; WD158 and WD161; WD159 and WD162; WD66 and WD155; WD66 and WD163; and WD66 and WD164. These primers produce a small E6 amplification product of approximately 250 base pairs in length.

The present invention also provides primers that are complementary to sequences in the HPV E1 region. These E1 primers can be used to amplify only E1 region sequences or can be used in conjunction with E6/E7 primers to amplify sequences from E6, E7, E1, and combinations of these three regions. These E1 primers are shown below in Table 9.

Table 9E1 POSITIVE STRAND PRIMERS

TYP01	20MER	A T G G C K G A Y C C T G M A G G T A C
TYP02	20MER	- - - - - - - - - - G A T T C - - - - - - -
TYP03	20MER	- - - - - - - - - - C C T T C - - - - - - -
TYP04	20MER	T G T A M W G G M T G G T T T T A T G T
TYP05	20MER	- - - - - - - - - - - - - - - - G A G - -
TYP06	20MER	- - - - - - - - - -- - - - - - - A T G - -

E1 NEGATIVE STRAND PRIMERS

TYN01	20MER	G T A C C T K C A G G R T C M G C C A T
TYN02	20MER	- - - - - - G A A T C - - - - - - - - -
TYN03	20MER	- - - - - - G A A G G - - - - - - - - -
TYN04	20MER	A C A T A A A A C C A K C C W K T A C A
TYN05	20MER	- - C T C - - - - - - - - - - - - - - -
TYN06	20MER	- - C A T - - - - - - - - - - - - - - -
TYN07	20MER	T C C A C T T C A G W A T T G C C A T A
TYN08	20MER	- - - - - - - - - - - - - - Y A - - - -

These E1 primers can be used in a variety of embodiments of the present invention. For instance, amplifications wholly within the E1 region can be performed using the primer pairs: (1) TYP01, TYP02, TYP03, and TYN07, TYN08; or (2) TYP04, TYP05, TYP06 and TYN07, TYN08. Note that TYP03 is similar to both TYP02 and TYP01 and can be omitted from the amplification. The E1 region is highly conserved among HPVs, however, and although typing of the sample is possible with an E1 amplification, typing is more readily accomplished when the E1 primers are used in conjunction with E6 or E7 primers, as follows. For instance, one can amplify the E6/E7 region using the following E1 and E6/E7 primer pairs: (1) WD72, WD76 and TYN01, TYN02, TYN03; (2) WD64, WD65 and TYN01, TYN02, TYN03; (3) WD72, WD76 and TYN04, TYN05, TYN06; (4) WD64, WD65 and TYN04, TYN05, TYN06; (5) WD72, WD76 and TYN07, TYN08; and (6) WD64, WD65 and TYN07, TYN08. In these latter amplifications, the entire E7 region is amplified. Thus, these amplification products can be detected with the E7 consensus probes depicted below:

TYP09	5' G A G C A A T T A G W A G A C
TYP12	- - - - - - - - - - A R Y - - -

Those skilled in the art recognize that the specific primers and probes disclosed herein are merely illustrative of the invention. For instance, because the primers and probes of the invention are single-stranded DNA molecules, and because the target DNA (HPV DNA in a sample) is double-stranded DNA, useful primers and probes of

the invention can be constructed merely by synthesizing primers and probes complementary to those specifically disclosed herein. The primers and probes of the invention can also be prepared to amplify and detect sequence variations within areas of HPV genomes other than those specifically exemplified herein.

Primers of the invention are generally 18 to 21 nucleotides in length and are designed to have a high degree of homology with HPV sequences. For instance, in the design of the genital HPV consensus primers of the invention, a high degree of homology with all five major genital HPVs (HPV types 6, 11, 16, 18, and 33) was required. For each region to be amplified, two regions of homology are required, one for negative-strand primers and another for positive-strand primers. To identify a homology region, viral sequences are compared. Once a homology region is identified, a consensus primer is designed. Degenerate bases can be used in the design to accommodate positions at which an individual virus varies in sequence from the homology sequence. As many degenerate positions are made as is necessary so that all viral sequences have fewer than three mismatches with the consensus primer. The degenerate positions are chosen so that the smallest number of degenerate bases accommodates the largest possible number of viral sequences.

If a particular viral sequence has a large number of mismatches with the consensus sequence, then a type-specific primer is made for that virus. The type-specific primer is mixed with the degenerate primer that was designed for other viruses to make the consensus primer. Any mismatches that are not accommodated by the degenerate positions in the primer should generally be located more than 3 bases from the 3' end of the primer. Likewise, any degenerate positions should be more than 3 bases from the 3' end of the primer.

Estimated minimum and maximum T_ms for a degenerate primer should be between 54 and 64 degrees C. T_ms are estimated by the non-empirical formula: each G or C contributes 4 degrees C to the T_m; each A or T contributes 2 degrees C to the T_m; and the T_m is the sum of the calculated values. Finally, primers should not be designed to span palindromes or repetitive sequences.

Consensus probe design is similar to consensus primer design, except that consensus probes generally do not contain as many mismatches as consensus primers. As a result, the T_m for a probe can be higher than the T_m for a primer. However, where a mismatch or degenerate position occurs with respect to the 3' end is not as critical for consensus probes as it is for consensus primers.

Type-specific probes are designed so that a given probe will generally have less than 75% similarity with sequences from HPV types distinct from that recognized by the probe. The type-specific probes are usually 18-20 nucleotides in length with estimated T_ms in the range of 58 to 64 degrees C.

Those skilled in the art also recognize from the present disclosure that the method of the present invention can be carried out in a variety of ways. The present method is applicable to any human papillomavirus and especially preferred for detecting and typing genital HPVs. The method can be used to detect isolate-to-isolate variation within a particular HPV type and can also be used to screen for significant changes in the HPVs present in a patient. In one embodiment of the invention, consensus primers to more than one region of HPV DNA will be used, ensuring that if any portion of the HPV genome has been deleted, other regions can still be detected. In a similar fashion, the typing of the amplified DNA can be done using a variety of type-specific probes that recognize different regions of the amplified DNA.

Those skilled in the art recognize that the present invention can also be used to detect HPV mRNA present in a sample. The expression of certain HPV mRNA species, particularly E6 and E7 mRNAs, may be indicative of the likelihood than an HPV infection will progress to carcinoma. To detect an HPV mRNA by the method of the present invention, the mRNA can be treated with reverse transcriptase in an appropriate reaction mixture to synthesize a cDNA molecule. The primer used in the reverse transcription reaction can be a consensus primer of the invention or can be a different oligonucleotide that hybridizes near the 3' end of the mRNA. This cDNA copy is then made into a double stranded DNA molecule, which can be detected and typed in accordance with the method of the present invention.

The consensus primers of the present invention can also be used to detect HPV types previously uncharacterized. For instance, HPV isolates 36 and 88, noted in Table 4, above, have never before been characterized. Thus, the consensus primers of the invention can be used to amplify DNA sequences of previously unknown HPV types. The amplified DNA can then be sequenced and the sequence data used to generate type-specific probes for use in the method of the present invention.

The examples provided below merely illustrate the invention and in no way limit the scope of the accompanying claims.

Example 1

Preparation of Clinical Samples for Amplification by the Polymerase Chain Reaction

Cervical and vulvar swabs and penile scrapes typically contain 10^3-10^5 cells. Cells were suspended in 2 ml of phosphate-buffered saline, pelleted by centrifugation, and the supernatant discarded. If cell suspensions were to be stored for a period of time before the test, antibiotics were generally added to the suspension (a commercially available antibiotic, such as 2X Fungi Bact Solution, is suitable for this purpose). The cell number was estimated, and if blood was present in the sample, the cells were suspended in 1 ml of TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) to lyse the red blood cells and then repelleted. About 10^2-10^4 cells were used per PCR reaction. The cells were first suspended in 100 µl of buffer containing 50 mM Tris, pH 8.5; 1 mM EDTA; 1% Laureth-12; and 200 µg/ml proteinase K. This mixture was incubated at 55 degrees C for about 1 hour, and then the proteinase K was heat-inactivated by incubating the mixture at 95 degrees C for 10 minutes. The samples were then treated in accordance with a standard PCR protocol with the consensus primers of the invention to amplify any HPV sequences present. Aliquots containing 10^2 - 10^4 cells were used per 100 µl of PCR reaction mixture.

Another method for preparing a cell suspension for PCR involves suspending the cells in 100 µl of deionized water and incubating the resulting suspension at 100 degrees C for 10-15 minutes.

Example 2

Extraction of Tissue from Paraffin

The method of the present invention will often be used to determine whether tissue sections in paraffin contain HPV. To prepare such a tissue section, typically 5-10 µM in width, for use in the present method, the following procedure was employed.

The tissue section was extracted twice with 1 ml of xylene or an alkane such as octane to remove the paraffin. Each extraction was for about one-half hour. The tissue section was then rinsed twice with 100% ethanol to remove the extracting agent and dried in a rotary evaporator. The section was then suspended in Taq buffer with detergents and proteinase K and treated as described in Example 1, except that the 55°C incubation was for 2-4 hours.

After heat inactivation of the proteinase K, the suspension was centrifuged to pellet debris, and about 1-20 µl of the supernatant were used for each PCR reaction.

Example 3

PCR Protocols

All PCR protocols were carried out using a Perkin-Elmer/Cetus Instruments Thermal Cycler instrument. A typical reaction mixture contained 50 pmoles of consensus positive-strand primer; 50 pmoles of consensus negative-strand primer; 2.5 Units of Taq polymerase; 10 µl of 10X PCR buffer (0.5 M KCl; 100 mM Tris, pH=8.5; 20-40 mM $MgCl_2$; 0.2 mM of each dNTP; about 10 µl of a clinical or paraffin sample; and deionized water to 100 µl.

PCR reaction times for use with clinical samples were as follows. The machine was heated to 72 degrees C before the samples were placed into the machine. The machine was then programmed to execute the following temperature changes: thirty cycles of 30 seconds at 95 degrees C, 30 seconds at 55 degrees C and 60 seconds at 72 degrees C; 5 minutes at 72 degrees C; and then hold at 15 degrees C.

For paraffin sections, the machine was programmed to execute temperature changes as follows: forty cycles of 50 seconds at 95 degrees C, 50 seconds at 55 degrees C, and 2 minutes at 72 degrees C; 5 minutes at 72 degrees C; and then hold at 15 degrees C.

If the PCR product was expected to be longer than 600 bp, the machine was programmed to execute temperature changes as follows: 1 minute at 72 degrees C; forty cycles of a 50 second ramp to 95 degrees C, 20 seconds at 95 degrees C, a 90 second ramp to 55 degrees C, 30 seconds at 55 degrees C, a 50 second ramp to 72 degrees C, and 3 minutes at 72 degrees C; 5 minutes at 72 degrees C; and then hold at 15 degrees C.

To determine by use of consensus probes if amplification had occurred, about 10 µl of the reaction mixture were added to 140 µl of deionized water and 50 µl of 4X denaturing solution (1.6 M NaOH and 100 mM EDTA). About 100 µl of this denatured solution were spotted onto a positively-charged, nylon Genetrans membrane using a BioRad dot-blot apparatus. The resulting dot was rinsed once with 200 µl of 20XSSC. The membrane was then removed from the blotter, air-dried, and exposed to ultraviolet light (with the DNA facing the light) to covalently attach the DNA to the membrane.

The membrane was pre-hybridized at least 45 minutes at 68 degrees C in a water bath. The pre-hybridization solution contained 6XSSC, 5X Denhardt's solution, and 0.5% SDS. Alternatively, the membranes can merely be rinsed with pre-hybridization solution. The pre-hybridization solution was decanted. About 10 ml of the pre-hybridization solution were added to 100 µl of 10 mg/ml denatured salmon sperm DNA

and to about 1 x 10^6 Cherenkov counts per mL of consensus probe (0.2 pmole). This solution was added back to the membrane, and the consensus probe was allowed to hybridize at 55 degrees C for at least 1 hour. After the hybridization, the hybridization mix was decanted and the membrane was quickly rinsed in a 30-55 degree C wash to remove excess probe. The wash solution was composed of 2XSSC and 0.1% SDS. The membrane was then washed in fresh wash solution heated to 55 degrees C. For this wash step the membrane was placed in a tray containing heated wash solution and the tray was placed in a 55 degrees C water bath for 10 minutes. This wash procedure was repeated once, and then the membrane was rinsed with a solution of 2XSSC and 0.1% SDS heated to 55 to 60 degrees C. An alternative wash procedure involves the same methodology but wash solutions at different temperatures, i.e., a 55 degrees C wash solution in the first two washes and a room temperature wash solution in the final rinse. The membrane was then air-dried and allowed to expose X-ray from 7 to 48 hours.

To determine the HPV type of amplified DNA, PCR reaction mixtures were hybridized to type-specific probes as described above. The only significant difference in the procedure was that the final wash of the filter was done at a temperature very near the Tm of the particular type-specific probe. Filters hybridized to different type-specific probes were not washed together.

Other modifications of the embodiments of the invention described above that are obvious to those of ordinary skill in the areas of molecular biology, medical diagnostic technology, biochemistry, virology, genetics and related disciplines are intended to be within the scope of the accompanying claims.

In the Claims

1. A method for detecting human papillomavirus (HPV) in a sample and typing the HPV, if present, comprising:

(a) treating the sample with consensus HPV primers, an agent for polymerization, and deoxynucleoside 5'-triphosphates under conditions such that an extension product of a consensus primer can be synthesized if HPV is present, wherein said consensus primers are a mixture of oligonucleotides that comprises at least a pair of primers sufficiently complementary to separate strands of HPV DNA to hybridize thereto so that the extension product synthesized from one member of said pair, when separated from its complementary strand, can serve as a template for synthesis of the extension product of the other member of said pair;

(b) separating the primer extension products, if present, from the templates on which the extension products were synthesized to form single-stranded molecules;

(c) treating the single-stranded molecules generated in step (b), if any, with the consensus primers of step (a) under conditions such that a primer extension product is synthesized using each of the single-stranded molecules produced in step (b) as a template;

(d) repeating steps (b) and (c) at least once;

(e) determining of amplification has occurred; and, if amplification has occurred,

(f) hybridizing a type-specific DNA probe to said amplified DNA; and

(g) determining if hybridization has occurred.

2. The method of Claim 1, wherein said HPV is a genital HPV.

3. The method of Claim 2, wherein step (e) comprises treating the reaction mixture prepared in step (d) under hybridizing conditions with a consensus probe and determining if hybridization has occurred.

4. The method of Claim 2, wherein said consensus primers can amplify transcriptional regulatory region sequences of said genital HPV.

5. The method of Claim 2, wherein said consensus primers can amplify L1 open reading frame sequences of said genital HPV.

6. The method of Claim 2, wherein said consensus primers can amplify E6 open reading frame sequences of said genital HPV.

7. The method of Claim 2, wherein said consensus primers can amplify E1 open reading frame sequences of said genital HPV.

8. The method of Claim 2, wherein said consensus primers can amplify E7 open reading frame sequences of said genital HPV.

9. The method of Claim 2, wherein said consensus primers can amplify E1, E6, and E7 open reading frame sequences of said genital HPV.

10. The method of Claim 2, wherein said type-specific probe can hybridize to transcriptional regulatory region sequences of said genital HPV.

11. The method of Claim 2, wherein said type-specific probe can hybridize to L1 open reading frame sequences of said genital HPV.

12. The method of Claim 2, wherein said type-specific probe can hybridize to E6 open reading frame sequences selected from the group consisting of E6 and E7 sequences of said genital HPV.

13. The method of Claim 3, wherein said consensus probe can hybridize to transcriptional regulatory region sequences of said genital HPV.

14. The method of Claim 3, wherein said consensus probe can hybridize to L1 open reading frame sequences of said genital HPV.

15. The method of Claim 3, wherein said consensus probe can hybridize to E6 open reading frame sequences of said genital HPV.

16. The method of Claim 3, wherein said consensus probe can hybridize to E1 open reading frame sequences of said genital HPV.

17. The method of Claim 3, wherein said consensus probe can hybridize to E7 open reading frame sequences of said genital HPV.

18. The method of Claim 4, wherein the entire URR region sequence of said genital HPV is amplified.

19. The method of Claim 4, wherein said primers are selected from the group consisting of FS10, JS15, JS16, FS17, and MY01.

20. The method of Claim 5, wherein said primers are MY11 and MY09.

21. The method of Claim 6, wherein said primers are selected from the group consisting of WD64, WD65, WD66, WD67, WD68, WD69, WD70, WD71, WD72, WD73, WD76, WD77, WD83, WD84, WD154, WD155, WD157, WD158, WD159, WD160, WD161, WD162, WD163, and WD164.

22. The method of Claim 7, wherein said primers are selected from the group consisting of TYN01, TYNO2, TYNO3, TYNO4, TYNO5, TYNO6, TYNO7, TYNO8, TYPO1, TYPO2, TYPO3, TYPO4, TYPO5, and TYPO6.

23. The method of Claim 9, wherein the entire E7 region sequence of said genital HPV is amplified.

24. The method of Claim 11, wherein said probe is selected from the group consisting of MY12, MY13, MY14, MY16, MY58, MY59, MY60, MY61, MY62, MY63, MY64, MY65, MY69, MY70, WD74, WD126, WD127, WD128,WD150, WD151, WD152, and WD153.

25. The method of Claim 12, wherein said probe is selected from the group consisting of WD78, WD79, WD80, WD81, WD82 WD102, WD103, WD104, WD132, WD133, WD134, WD165, WD166, WD167, WD168, and WD169.

26. The method of Claim 13, wherein said primers comprise at least one E1 primer and at least one E6 primer.

27. The method of Claim 14, wherein said consensus probe comprises a probe selected from the group consisting of FS10, MY18, MY19, MY39, MY55, MY56, MY57, MY66, MYXX, and WD147.

28. The method of Claim 15, wherein said consensus probe comprises a probe selected from the group consisting of WD64, WD65, WD83, and WD84.

29. The method of Claim 17, wherein said consensus probe comprises a probe selected from the group consisting of TYP09 and TYP12.

30. Oligonucleotides useful for detecting HPV DNA on a sample selected from the group consisting of: FS10, JS15, JS16, FS17, MY01, MY11, MY09, WD64, WD65, WD66, WD67, WD68, WD69, WD70, WD71, WD72, WD73, WD76, WD77, WD83, WD84, WD154, WD155, WD157, WD158, WD159, WD160, WD161, WD162, WD163, WD164, TYN01, TYNO2, TYNO3, TYNO4, TYNO5, TYNO6, TYNO7, TYNO8, TYPO1, TYPO2, TYPO3, TYPO4, TYPO5, TYPO6, MY12, MY13, MY14, MY16, MY58, MY59, MY60, MY61, MY62, MY63, MY64, MY65, MY69, MY70, WD74, WD126, WD127, WD128,WD150, WD151, WD152, WD153, WD78, WD79, WD80, WD81, WD82 WD102, WD103, WD104, WD132, WD133, WD134, WD165, WD166, WD167, WD168, WD169, MY18, MY19, MY39, MY55, MY56, MY57, MY66, MYXX, WD147, WD64, WD65, WD83, WD84, TYP09, and TYP12.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/03747

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 Q 1/70 // C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC5	C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Brief Definitive Report, Vol. 167, 1988, Darryl K. Shibata et al: "Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. ", see page 225 - page 230	1-30
X	WO, A1, 88/06634 (THE UNIVERISTY OF SYDNEY) 7 September 1988, see the whole document	1-30
A	EP, A2, 0235004 (INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE(INSERM)) 2 September 1987, see the whole document	1-30

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th December 1989	10. 01. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A2, 0192001 (INSTITUT PASTEUR) 27 August 1986, see the whole document -- --------	1-30

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 89/03747

SA 31674

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 08/11/89
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 88/06634	07/09/88	AU-D-	13938/88	26/09/88
EP-A2- 0235004	02/09/87	FR-A-	2593828	07/08/87
		JP-A-	62248492	29/10/87
EP-A2- 0192001	27/08/86	FR-A-B-	2578267	05/09/86
		JP-A-	61216700	26/09/86
		FR-A-B-	2581655	14/11/86